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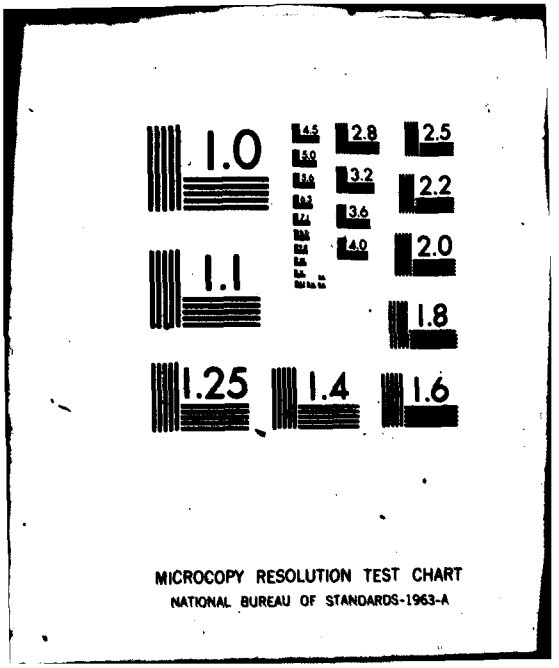
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Budget justification:

Nine per cent salary increments are requested over current salary levels after 1 July 1981. Fringe is calculated at 20.5 per cent for faculty and 17.5 per cent for non-faculty salaries.

Equipment requested is not available from other sources within Yale University. The vertical laminar flow safety cabinet is needed during procedures which create aerosols, to protect personnel working with CDC class 3 viruses and unknown viruses from possible infection .

1. GENERAL STATEMENT

This contract proposal is submitted with the request that it be funded through the Office of Naval Research. Until July, 1977, the Naval Medical Research and Development Command supported jointly with the U.S. Army, the World Arbovirus Reference Center at Yale University through Contract DADA 17-72C-2170. The Contract is now funded solely by the Army with $63,899 total direct costs for the period 7/1/80 to 6/30/81; additional support for the World Reference Center includes $69,039 total direct costs from the National Institute of Allergy and Infectious Diseases, $15,125 from the Australian government, and $12,150 from WHO for the current year. The studies herein proposed complement directly those of two naval officers who conduct research at Yale University in collaboration with faculty of the Yale Arbovirus Research Unit.

2. SUMMARY OF PROGRESS TO DATE (For detailed progress reports see appendix 1, Annual report for 1979 of Yale Arbovirus Research Unit.)

i. Rapid and early detection of arbovirus antigens and antibody.

The enzyme-linked immunosorbent assay (ELISA) was extended to the detection of antibodies to Japanese encephalitis (JE), yellow fever (YF), and Rift Valley fever (RVF) viruses in human sera (previously collected for diagnostic or survey purposes). The YF and JE detection experiments were limited in scope, but showed that in both convalescent and post-vaccination sera the ELISA yielded results not reproducibly in agreement with the hemagglutination-inhibition (HI) and neutralization tests (NT). This parallels our previous observations with dengue viruses, and indicates that the ELISA test of antibodies to flaviviruses is not as straightforward as with other viruses and needs further development. The RVF ELISA was developed from inactivated antigens. Currently we are employing this test to determine the time of onset and magnitude of antibodies in serial sera collected from patients with documented RVF. This collection of sera has been exhaustively analysed by NT, HI, complement-fixation (CF), and indirect-fluorescent antibody (IFA) tests, and tested for IgG and IgM antibody response. To date, ELISA appears to parallel the NT antibody response, but further comparisons, and IgG and IgM antibody detection are in progress.

Parameters affecting the ELISA test also have been studied. Increasing the purity of viral antigen improves the sensitivity and specificity of the test,

but the process of attaching the antigen to the solid phase (polystyrene plate) remains undefined and variable. We have developed an ELISA utilizing antigen covalently bound to a solid phase (either agarose or polyacrylamide microspheres). Our preliminary results show these antigen-spheres can be used as antigen in the HI, ELISA, or IFA tests (and presumably will function in the CF and radioimmunoassay tests). Current studies are designed to perfect this multitest antigen and determine its ability to be lyophilized, reused, and/or employed in a polyantigen ELISA test.

Antigen detection studies using ELISA have continued on a bunyavirus (Guaroa) model system. The most sensitive procedure proved to be a four-layer sandwich method as opposed to a double-antibody sandwich method. Employing this procedure, as little as 6.6×10^4 plaque forming units (pfu) of virus were detected. The procedure detected Guaroa virus in a pool of mosquitoes containing 2 infected and 50 uninfected insects.

Studies comparing the IFA response with the NT, HI, ELISA, and CF tests indicate that IFA antibodies (both IgG and IgM) are the first to be detected during recovery from RVF. They also appear prior to onset of clinical signs of RVF encephalitis and RVF retinitis, and thus could be used for rapid diagnosis of these clinical syndromes. However, cross-IFA tests (see below) showed extensive cross-reactions with other phlebotomus fever (PHL) group viruses commonly causing human disease in Africa and the Middle East. The ELISA might prove less cross-reactive.

ii. World Arbovirus Reference Center

Virus Identification. Two viruses referred by NAMRU-3 and isolated from Ornithodoros capensis and one virus from Rhipicephalus sanguineus ticks collected in the Seychelle Islands have been tentatively identified as Soldado Rock virus. Another virus has been isolated from Hyalomma A. anatolicum ticks collected in Bahrain and is currently being identified.

Four different viruses from Uganda were studied. An isolate from mosquitoes was identified as Pongola; an isolate from a fruit bat and from 4 human febrile cases was related to Yogue virus and is a new member of that group; an isolate from Amblyomma variegatum ticks was bunyavirus-like by electron microscopy but did not react serologically with other African viruses tested; and another isolate from human serum was chikungunya virus.

An interesting virus from brain of a South African dog suspected of rabies was West Nile virus, and another South African isolate from mosquitoes did not react by CF with antigens or sera of over 400 viruses, and is presumably new to science.

An agent from Ixodes uriae ticks of Macquarie Island, Australia was a new member of the group B tick-borne encephalitis complex, the first such member from south of the equator. Umbre virus of the Turlock group was identified for the first time from mosquitoes of Australia. Another virus from the serum of a patient in Fiji was closely related to Ross River virus, the cause of epidemic polyarthritis and rash.

A virus from soft ticks collected near Cape Frehel, France was a sub-type of Soldado Rock virus.

Four viruses from mosquitoes collected near Lake Nasser, Egypt were identified as Sindbis virus.

Six viruses from mosquitoes of New Caledonia were group B agents, not yet identified to type; three additional isolates from mosquitoes and birds have been established but not yet grouped.

An isolate from human serum from a febrile patient bled in the Netherlands was Colorado tick fever virus. The patient had vacationed in the western U.S.A. and returned sick to Holland where he removed a tick from himself. This is an example of long-distance transport of a human viral pathogen.

An agent recovered from Dermacentor variabilis ticks in Canada was negative in testing with sera to 87 viruses; another agent from Ornithodoros maritimus ticks collected in herring gull nests in Morocco was identified as a virus closely-related to Chenuda of the Kemerovo group.

Virus taxonomy. Members of the Sakhalin serogroup were shown to be related by HI and fluorescent focus neutralization tests, and to belong to the Nairovirus Supergroup. Multiple viruses in the supergroup and corresponding reference sera were supplied to the University of Alabama where studies of RNA and proteins showed that these viruses formed a new genus in the family Bunyaviridae.

Serologic surveys. Surveys were conducted with sera from Ghana, Cameroon, Sudan, and Liberia using the IFA test. Sera positive for Ebola and Marburg antibody were found in Ghana. The Ebola positive sera were collected one year before the first recorded Ebola outbreak. Five of 41 sera from Cameroon were positive also for Ebola and 16/196 human sera from Liberia had Lassa virus antibody.

Sera from Sudan (see below) contained IFA antibodies to West Nile, group A (probably Sindbis and chikungunya), Rift Valley fever, group Bunyamwera (probably Bunyamwera, Germiston, and Ilesha), Tataguine, Quaranfil, Bwamba, Tahyna, Bangui or Zinga, and Sicilian sandfly fever viruses. Antibody to Ebola and Lassa viruses was also found.

Development of techniques and models. In further development of the use of RNA purification and gel electrophoresis, we found that Colorado tick fever (CTF) virus contained 12 segments, 2 more than found in any other orbivirus. This surprising result may mean that CTF virus, believed to be an orbivirus, is in a different genus of the Reoviridae family. A presumably new virus from NAMRU-5, Ethiopia was confirmed to be an orbivirus with 10 RNA segments by the polyacrylamide gel electrophoresis technique.

The IFA test has proved rapid, sensitive, and for some families of viruses, widely cross-reactive. This test offers the possibility of rapid, inexpensive virus identification and survey for antibody. The technique was developed and tested for members of a number of serological groups of arthropod-borne viruses.

Within arbovirus groups A, Bunyamwera, and PHL, the test was broadly cross-reactive and as such was useful as the first test of broad serological surveys.

A plaque assay system and a plaque reduction neutralization test (PRNT) were developed for most members of the Nairovirus supergroup (which includes Crimean-Congo hemorrhagic fever, Nairobi sheep disease, Dugbe, and Soldado). The relationships between viruses in this supergroup are currently being evaluated in the PRNT.

The following cell lines were adapted for growth on bovine calf serum instead of bovine fetal serum: Vero, LLC MK_2, BHK-21/C13, L-929, and CER. Adaptation was necessary because of the inability to obtain bovine fetal serum from commercial sources.

Distribution of reagents . The reference center distributed 518 ampoules of reference sera, viruses, and antigens during 1979, and 391 ampoules during the first half of 1980; mosquito and vertebrtate cell lines were also distributed. During 1980 new seed stocks for over 30 different viruses were prepared in Vero cell culture, with over 770 ampoules added to the reference bank.

iii. Serological studies of the Sudanese human population.

Since Sudan lies in a transitional climatic zone between equatorial and northern Africa, it is likely the many arthropod-borne viruses which circulate in both areas are disease problems in Sudan. Over 800 sera were collected from male and female outpatients of all ages seen at the Khartoum Hospital, and from mostly male military recruits from all areas in the country. Five hundred sera have been screened for antibodies using the IFA and the CF tests. Antigens were those arthropod-borne viruses which have been implicated as human pathogens in equatorial, northern and southern Africa. The percentages of antibodies obtained to date are: West Nile 32.1, polyvalent alphavirus antigen (Sindbis, chikungunya, and o'nyong-nyong) 3.5, Rift Valley fever 3.2, polyvalent Bunyamwera (Bunyamwera, Germiston, Ilesha) 5.7, Tataguine 18.8, Quaranfil 1.0, Bwamnba 3.4, Tahyna 1.0, polyvalent Bangui and Zinga 1.5, and Sandfly Sicilian 22.3. Other antibodies detected at less than 1.0% were Gordil, Gabek Forest, sandfly fever Naples, Arumowot, Shuni, Saint-Floris, Nyando, and Dugbe. Tests for antibodies to Thogoto, Wad Medani, and Malakal viruses were negative.

In conjunction with an ongoing survey for antibodies to Lassa, Marburg, and Ebola viruses in equatorial Africa, sera from southern Sudan were also screened for these antibodies. Sixty-nine sera have been tested to date; 13 were positive with the trivalent antigen slide. Four of these positive sera were tested with monovalent slides; two were positive only for Ebola, one for both Ebola and Lassa, and one gave questionable results. Further testing is in progress to characterize the positive results and to evaluate sera from other geographic areas within Sudan.

iv. Phlebotomus fever group protein/antigenicity studies.

Study of the antigenic determinants of members of this group requires cell culture systems for production and assay of these viruses. These systems were

developed and a new, simpler plaque assay was perfected which does not require either DMSO or methylcellulose as overlay. The IFA technique was used to study cross-reacting proteins among members of the PHL group. Cross-reactions were detected which have not been seen in the Nt, HI, or CF tests (e.g. antisera to RVF virus reacted with sandfly fever Sicilian-infected cells) indicating these viruses probably share common antigenic sites on non-structural proteins induced during intracellular virus growth. Unfortunately, this might complicate diagnosis since the IFA is commonly used for diagnosis of human illness. Animals are being immunized for production of hybridoma monoclonal antibodies to PHL group viruses, which will aid in distinguishing and studying antigenic determinants within this group.

v. Study of the cause of RVF disease syndromes in human beings.

The encephalitic syndrome resulting from RVF virus infection occurs after the acute febrile phase of the disease and evidence suggests an immunological basis for this manifestation. Results of experiments performed in the U.S. Army using animal model systems indicate that previous exposure to a serologically related virus increases the probability of development of RVF encephalitis. The IFA test was used during this reporting period to study a group of sera from Egyptian patients with RVF encephalitis. Testing was done with RVF and a battery of other PHL group viruses to determine the patients' previous exposure to PHL viruses. These patients did not show a pattern of early heterologous or broadened cross-reaction which might indicate pre-existing antibodies to PHL viruses. However, the IFA test might not detect low levels of antibody and our initial results must be interpreted cautiously until ongoing Nt tests can establish if in these patients a previous PHL virus infection predisposed them to RVF encephalitis.

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3. REVIEW OF SCIENTIFIC BACKGROUND

i. Rapid and early detection of arbovirus antigen and/or antibody.

The current techniques to make a specific diagnosis of arbovirus infection in man involve isolation and identification of the virus or detection of a rise in antibody levels between acute and convalescent sera. These are usually lengthy procedures which require sophisticated and well-equipped laboratories.

Experience with the classical techniques of arbovirus antibody measurement indicates that antibodies are detected earliest by the neutralization test followed by the HI and then by the CF tests (1, 2, 3, 4, 5). A recent study of Central European tickborne encephalitis in Croatia, Yugoslavia (6) between 1963 and 1974 showed that 21% of 807 sera taken from patients late in the first week and early in the second week of illness were negative by CF while only 2% were negative by HI. The classical techniques are highly reliable and most can be done under field conditions without expensive apparatus; however the need is evident for more sensitive and more rapid techniques if such can be found without sacrificing reliability. The fluorescent antibody (FA) test, the enzyme-linked immunosorbent assay (ELISA), and the enzyme-linked fluorescent assay (ELFA) offer promise of greater sensitivity and are proposed here for additional research development and application to early and rapid diagnosis of arbovirus infection.

Enzyme linked immunosorbent assay. The ELISA test was devised by Engvall and Perlmann in 1971 (7). An antigen or antibody is conjugated to an enzyme allowing quantitative assay when a substrate is added and the reaction is read by color change. The test can be adapted for detection of a virus in patient

serum or other materials, for measurement of antibody rise in acute and convalescent sera, and for identification of unknown antigens using reference sera. It is extremely sensitive; the enzyme-antigen conjugate can be stabilized over a long time period and the test does not require the equipment needed for the FA and RIA methods. ELISA has been applied widely in parasitic diseases such as malaria, African trypanosomiasis, Chagas, amebiasis, toxoplasmosis, Babesiosis, leishmaniasis, and schistosomiasis (8) as well as in viral diseases such as measles, rubella, rotaviruses, and cytomegalovirus (9,10,11,12). Its adaptation to arboviruses was accomplished through this contract (13).

Enzyme linked fluorescent assay. Recently studies revealed that by substituting a substrate which yields either a fluorescent or radioactive product the sensitivity of the ELISA for antigen and antibody detection can be increased by 1000-fold (14,15). Although there are limitations in using radioactivity (see below), the availability of fluorimeters makes the ELFA a diagnostic tool which has already shown promise for the rapid detection of virus in nasal swabs from patients with influenza infection (16). Our future studies will perfect the ELISA, extend it to other virus systems, and establish the diagnostic applicability of the ELFA.

For the detection of antigen, both the ELISA and ELFA require purified antibody to coat the solid phase and bind the virus. Studies have shown that the sensitivity of antibody detection is improved by addition of a second antibody to the ELISA system (17). Presumably, the development of monospecific antibodies with strong avidity for the virus would increase the specificity and sensitivity of these tests. Although purification and fractionation of antibodies using immunosorbent affinity chromatography columns is an approach to obtaining these antibodies, it has provided unsatisfactory yields in our laboratory. A more successful approach might be the production of monospecific antibodies using the hybridoma technology (18,19,20).

The high sensitivity of RIA suggested its applicability to the arthropod-borne group of viruses. However, the comparable sensitivity of the ELFA (14) and the increasing problems with safety and disposal of radioactive material have cast doubts as to the wisdom of continuing the development of this type of assay.

Fluorescent antibody test. Successful use of the FA test has been reported with the arboviruses Colorado tick fever (21) and Crimean hemorrhagic fever (22), as well as with other viruses such as rabies (23), LCM (24), and Lassa fever (25). Technological advances which have facilitated the use of FA for serologic diagnosis include the commercial availability of incidence fluorescent light microscopes at reasonable prices, and the development of multi-chambered microscope slides and of teflon-coated water-repellent spot slides which allow multiple serum samples to be processed simultaneously. The demonstration of antigen by FA directly in patient materials such as blood (21) and skin biopsies (26) allow almost immediate diagnoses to be made, at least in these specialized cases. In addition, studies in this laboratory with Lassa fever as well as those at CDC (25) showed that the FA test detects antibody earlier and longer than the CF test and is thus a more sensitive diagnostic technique. The same conclusions appear to be true for Rift Valley fever infection and probably other arthropod-borne viruses.

ii. World Arbovirus Reference Center. The Yale Arbovirus Research Unit serves as the arbovirus reference center for the world. Any virus suspected of being biologically transmitted by arthropods is accepted for identification and characterization. A collection of over 400 already characterized type viruses is maintained with complementary sera and diagnostic antigens. Studied only with inactivated reagents are the viruses of Nairobi sheep disease, African horse-sickness, Rift Valley fever, Marburg, African swine fever, Lassa, and Ebola, because of their hazard to man and domestic livestock; virtually all others are represented.

The Unit and the World Reference Center (designated WHO Collaborating Center for Reference and Research) are direct outgrowth of the Rockefeller Foundation program of a worldwide network of laboratories for the investigation of the role of arthropod-borne viruses in producing disease in human beings and animals and for the study of the mechanisms by which these viruses are maintained in nature. When this program was initiated in the early 1950's, less than 28 arboviruses had been described and only a few, such as yellow fever, the encephalitides, and dengue were known to cause serious disease in human beings. Concurrent with the initiation of the Rockefeller Foundation program was the establishment of laboratories by the U.S. Navy, U.S. Army, the U.S. Public Health Service and several foreign governments. This network of field laboratories relied on the Foundation's central virus laboratory, located at The Rockefeller Institute in New York, and since 1964 on the Yale Arbovirus Research Unit, for the refined facilities needed to provide final identification and biological correlation of the arboviruses isolated. This service is a vital one in that it provides prompt analysis of disease outbreaks and identification of new virusees to government health services, regional epidemiological centers, and worldwide epidemiological intelligence services of the World Health Organization and U.S. and foreign government agencies. It also serves the world research community with basic certification of arboviruses and reagents. The identification and supply of reagents are gratis and proffered willingly since a world reference center must function freely without inhibiting any collaborator in order to ensure receipt of all new viruses for a complete reference collection.

The reference center is only part of the overall activity of the Unit, which also has strong teaching and research components. The research interest and capability is essential to the reference function because the collection cannot be maintained efficiently without avid use. In turn, the reference component generates research ideas, and as projects evolve, these have been (and will be) subjects of separate research grant proposals to funding agencies.

The methods utilized are those which have proved reliable in recent years. The antigenic and morphogenic classification systems are employed because these represent stable characteristics for most of the arboviruses. The Yale Arbovirus Research Unit is currently the only laboratory in the world with the relatively complete collection of reference viruses needed to characterize fully new arboviruses and to respond to unusual and exotic diagnostic problems.

During the 7 years between 1972 and 1979, virus and/or antiserum to 764 strains were received from 39 countries. Serological testing with 31 grouping ascitic fluids and screening with more than 200 antigens indicated that at least

36 of these were ungrouped viruses new to science. Other new viruses belonged to 20 different arbovirus groups including groups A, B, Kemerovo, Sakhalin, Palyam, Uukuniemi, Yogue, Turlock, Rabies, Dera Ghazi Khan, Boteke, Changuinola, phlebotomus fever, Tete, Nairobi sheep disease, Bandia, Simbu, Colorado tick fever, Barur, Sawgrass, and Eubenangee. Revision of these groups by the complement-fixaton and other serological tests was carried out. New geographic records were established for 41 known arboviruses.

New techniques or applications were developed. Aedes albopictus cells were used for primary isolation of arboviruses. Methods for detecting contaminants of mosquito cells, for removing non-specific arbovirus HA inhibitors, for VEE RNA-RNA homology studies, for making large quantities of interferon, for type-specific diagnosis of California encephalitis, orbivirus, and VEE subtypes, and for adapting ELISA to arboviruses were developed.

In the same 7 year period, ten epidemics were investigated virologically or serologically, including one of Lassa fever, Rift Valley fever in Egypt and an outbreak of probable Semliki Forest encephalitis in African horses. Sero surveys of West Africa, Turkey, British Honduras, Iran, and Indonesia indicated widespread prevalence of arbovirus antibody. The reference center distributed 2,191 ampoules of virus, 1,747 of antigen, and 2,736 of immune reagents. In addition 178 Aedes cell cultures were sent to U.S. and foreign laboratories.

iii. Survey of Sudanese and Egyptian sera for antibody to arboviruses. It is likely the many arthropod-borne viruses which circulate in northern and southern African areas are disease problems in Sudan. Few studies have been undertaken in this geo-politically important country. During 1979 and 1980, over 800 sera were collected from recruits in Sudan. These represent collections from most areas of Sudan, and age, birthplace, and district of residence are available for all. Our survey is the first phase of a study to determine the impact of arthropod-borne viruses on human beings and animals in Sudan. In addition, since Sudan may act as a tunnel for the movement of viral disease from sub-Saharan Africa to Egypt and beyond, survey for other arboviruses may give clues as to what diseases to be alert for.

Our initial studies have revealed that antibodies to Rift Valley fever virus are present in less than 5% of the population. Additional results obtained during this contract year have expanded our knowledge of distribution of a number of viruses (e.g., Ebola and Lassa, see progress report), and implicated as possible pathogens some unstudied viruses which appear in relatively high frequencies in the population (e.g., Tataguine, Zinga, and Bangui).

Collections of Egyptian sera from the 1977-78 Rift Valley fever epidemic area and from patients recovering from RVF are proving a valuable source of material for studies of the disease itself and of methods for rapid diagnosis. They continue to represent an important resource for determining the distribution of infections we have uncovered as prevalent in the Sudan.

iv. Phlebotomus fever group virus proteins, their antigenic determinants, and the relationship of proteins to intra-group serologic cross-reactions and to virulence.

There are 23 members (including Rift Valley fever) of the phlebotomus fever serogroup. Those which have been studied have 3 RNA segments (large, middle-sized, and small). These viruses have morphologic and physical characteristics which are similar in all respects to members of the genus Bunyavirus in the family Bunyaviridae (27). The L, M, and S RNA's of bunyaviruses code for 4 virion polypeptides. The L segment is believed to code for the polymerase; the M segment for 2 surface glycoproteins, and the S segment for the nucleocapsid protein. Nonstructural proteins have not been identified, although they may exist.

Robeson et al. (27) showed that Karimabad viral polypeptides can be resolved by electrophoresis in continuous 8% polyacrylamide gels and in discontinuous slab-gels. They also showed that the 2 glycoproteins were separable in polyacrylamide gels only after dissociation in SDS in the absence of beta-mercaptoethanol.

Alternate methods of separating polypeptides are available which do not degrade the proteins. The electrofocusing technique separates by charge and has been extensively used in the study of togaviruses (28,29). Polypeptides thus separated can be used to immunize mice for the production of antibody to viral sub-units; lymphocytes of such mice can also be used to produce monoclonal antibody by the hybridoma technique. In addition, monoclonal antibody produced by lymphocytes from mice immunized with whole virus will react specifically with antigenic determinants on viral sub-units (30,31,32,33), and different clones will have specificity for different sub-units.

The combined use of polypeptide purification and hybridoma techniques provide the means with phlebotomus fever group viruses, 1) to map the antigenic determinants relating them to specific polypeptides, 2) to relate the CF, HI, ELISA, RIA, FA, and neutralization reactions to corresponding polypeptides, 3) to relate serologic reactions to virulence in mice, 4) to relate specific polypeptides to the serologic cross-reactions seen between Rift Valley fever virus and other phlebotomus fever group viruses in the diagnostic setting and in serosurveys, and 5) to produce monoclonal antibody for diagnostic use by NAMRU field laboratories.

v. Studies of arbovirus infections in Indonesia.

Indonesia represents a strategic area of the world where arboviral disease is believed to be responsible for significant morbidity in the human population. Sera and unidentified viruses collected by NAMRU-2 field staff in Indonesia have been received at Yale for study. These materials include a) mosquito isolates suspected of being Japanese encephalitis (JE) virus from Kapuk, a hyperendemic JE area, b) viruses and paired acute and convalescent sera from fever cases, c) survey sera from animals and persons from nearly every island group of the Indonesian Archipelago, and d) viruses recovered in Indonesia from fever patients of Central Java and from bats and mosquitoes caught in recently deforested transmigration areas. Study of these materials should enhance knowledge of geographic distribution of arboviruses, their disease patterns, and their epidemiology.